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EFFECTIVENESS OF RHIZOBIAL STRAINS ON ARROWLEAF CLOVER GROWN IN ACIDIC SOIL CONTAINING MANGANESE

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Summary—Arrowleaf clover (*Trifolium vesiculosum* Savi) is an important forage legume in the southeastern U.S. but growth is limited on acidic soils. A glasshouse experiment was undertaken to assess growth of arrowleaf clover dependent on dinitrogen fixation under acidic soil conditions. Sixteen strains of *Rhizobium leguminosarum* biovar *trifolii* were evaluated for effectiveness with arrowleaf clover and survival in soil at pH 5, 5.6 and 6.8. Effectiveness, as determined by dry matter yield, ranged from a high of 216 to a low of 80 mg plant^{-1} at pH 6.8 and from a high of 110 to a low of 60 mg plant^{-1} at pH 5.6. Effectiveness at pH 5 was similar to effectiveness at pH 5.6. There was a significant rhizobial strain by pH interaction on plant dry matter yield. Yield of arrowleaf clover dependent on mineral N declined only 22% due to reduced soil pH. Inoculated plants grown at pH 6.8, 5.6 and 5 contained tissue Mn concentrations of 194, 524 and 768 mg kg^{-1}, respectively. Uninoculated plants supplied mineral N accumulated similar quantities of Mn. The population size of rhizobia in soil 5 months following harvest of the clover was dependent on the strain and pH, but populations of all strains exceeded 100 rhizobia g^{-1} soil.

INTRODUCTION

Arrowleaf clover (*Trifolium vesiculosum* Savi) is an important forage legume in the southeastern U.S. where many of the soils are acidic. Growth of legumes on acidic soils may be retarded due to N deficiency, since soil acidity is known to inhibit rhizobial survival, root colonization, infection and nodule activity (Munns, 1978). Germination of arrowleaf clover seed is reduced at pH 5 or lower (Evers, 1985) and yield is reduced by nearly 40% as compared to plants grown at pH 6 (Hoveland *et al.*, 1969). It appears that it is not practical to grow arrowleaf clover on soils more acidic than pH 5. Poor growth of clover on acidic soils may be due to available Al or Mn. Toxic concentrations of Al are not likely to be present unless the pH is below 5 (Munns, 1978), however, toxic concentrations of Mn may be present (Foy, 1984).

Survival and growth of *Rhizobium leguminosarum* biovar *trifolii* is influenced by pH but the effect is minimal in soil above pH 5 (Holding and Lowe, 1971). Rhizobia are relatively tolerant to Mn concentrations that are much higher than would be expected in soil solution (Holding and Lowe, 1971; Keyser *et al.*, 1979), but dinitrogen fixation is reduced by large concentrations of Mn in plant culture solutions (Vose and Jones, 1963; Lowe and Holding, 1970). For white clover (*T. repens* L.) and beans (*Phaseolus vulgaris* L.) the strain of rhizobia influences the sensitivity of the symbiosis to Mn (Lowe and Holding, 1970; Dobereiner, 1966). The strain of rhizobia also interacts with pH in determining dry matter production of cowpea [*Vigna unguiculata* (L.) Walp.] (Keyser *et al.*, 1979).

Our purpose was to determine the ability of rhizobial strains to promote growth of arrowleaf clover when grown on a soil containing potentially-toxic concentrations of Mn and to compare the growth with plants provided mineral N.

MATERIALS AND METHODS

Effectiveness of symbiosis

The experiment was a two-factor factorial with 14 strains of rhizobia and three pH regimes. Uninoculated controls with and without added N were included as inoculation treatments. There were four replicates of each treatment combination.

Five of the 14 strains of *R. leguminosarum* biovar *trifolii* we had isolated from nodules of clover grown on two acidic soils in Texas (6A, 6B, 6C, 9A and 9B). Strains RP115-5, RP115-9 and RP117-1 were obtained from Dr Tom Wacek, Research Seeds, Urbana, St Louis, MO. Strains 162Y13, 162Y14, 162Y17, 162K13, 162X68 and 162X97 were obtained from Dr S. Smith, The Nitragin Co. Inc., Milwaukee, WI. Strain TA1 was obtained from CSIRO, Australia. Rhizobia were grown on yeast extract mannitol agar slants (Weaver and Frederick, 1982) and suspended in 10 ml sterile water for use as inoculum.

Soil, having a pH of 5.6, was collected for the experiment from the A and E horizons (0–30 cm) of a Bowie variant (Glossic Paleudalf, fine-loamy, siliceous, thermic), located in a pine forest in Houston County, TX. The soil was amended with plant nutrients by adding 544 mg KH_2PO_4, 348 mg K_2SO_4, 4 mg Zn, 2 mg B, 0.3 mg Cu and 0.1 mg Mo kg^{-1} soil. To obtain pH regimes 5, 5.6 and 6.8 the amended soil was divided into three batches. One batch (pH 5) was acidified by adding 50 μl conc. H_2SO_4 kg^{-1} soil, and another batch (pH 6.8) was amended with 0.4 g $CaCO_3$ kg^{-1} soil to increase the pH. Each batch was mixed for 30 min using a cement mixer. The third batch (pH 5.6) was not altered. The soil was kept moist for 3 wk by which time the pH had stabilized. Chemical characteristics (Table 1) of the amended soils were determined by the Texas Agricultural Extension Service Soil and Plant Analysis Laboratory. Soil pH was determined in a 2:1 w/w mixture of water and soil. Soil P, K, Ca, Mg and Na were determined in ammonium acetate extracts. The ammonium acetate solution contained 25 mM H_4 EDTA, 1.4 M ammonium acetate and 1 M HCl. P was determined by the molybdate blue method (Olsen and Sommers, 1982), Na and K were determined by flame photometry, and Ca and Mg were determined by atomic absorption spectrophotometry (AAS). Soil Zn, Fe, Mn and Cu were determined by AAS on DTPA extracts of soil. The DTPA solution was 0.1 M triethanolamine, 5 mM DTPA, 60 mM HCl and 10 mM $CaCl_2$.

Two weeks before the experiment the soil was fumigated with methyl bromide for 72 h to kill indigenous rhizobial populations. The soil was stirred daily during the following week to facilitate aeration and release of the methyl bromide.

One litre pots were filled with 1.4 kg of the appropriate soil and 12 germinating arrowleaf clover seeds were planted. One drop of a rhizobial suspension containing approx. 10^5 cells ml^{-1} was added to each seed at planting. Ten days after planting and growth in a glasshouse the seedlings were thinned to 9 pot^{-1}. Water loss from each pot was determined gravimetrically and distilled water was added daily to return the soil moisture content to field capacity. To facilitate watering and reduce the possibility of cross contamination due to splashing, water was added through a PVC pipe (12.5 × 2.5 cm dia) that was inserted 10 cm into the soil near the centre of the pot. The uninoculated plus N treatments were watered with 4 mM KNO_3 daily. The uninoculated no-N treatments received only water. Glasshouse temperature was maintained between 20 and 28°C. Effectiveness of the symbiosis was defined as the quantity of dry matter accumulated in plant tops of inoculated plants minus that of the uninoculated control plants with no supplemented mineral N. Plant tops were harvested 52 days after planting and were dried at 65°C in a forced draft oven to constant weight. The roots from two pots of each treatment were examined for nodulation following harvest of plant tops. The remaining two pots were used for evaluation of rhizobial survival and roots were left intact.

Mn content was measured in plant shoots from selected treatments. Because of the low dry matter yield at pH 5 and 5.6, plants inoculated with strains 6A and 162X68 were combined as one sample and plants inoculated with strains 162Y14 and TA1 were combined as a second sample. The plus mineral N uninoculated plants was a third sample. The plants were oven-dried for 24 h, ground in a Wiley mill to pass a 850 μm screen and analyzed for Mn by atomic absorption spectrometry (Parkinson and Allen, 1975).

Survival of rhizobia

The effect of soil pH on survival of rhizobia was assessed after the harvest of the clover using the most probable number method (Weaver and Frederick, 1982). Two pots from each treatment containing soil and roots were stored in the glasshouse for 1 month to allow nodules to decompose. Strain 162X97 was not included due to an error during harvesting. After 1 month the roots were removed and the soil in each pot was mixed. A 300 g sample was removed and moistened by adding 30 ml of water. The sample was stored at 25°C in a plastic bag for 4 months, after which the number of rhizobia was determined. A sub-sample of 10 g was removed from each bag and used to make 10-fold serial dilutions which were used to inoculate arrowleaf clover seedlings in growth pouches. Population data were converted to log numbers for analysis of variance. The number of rhizobia was not determined immediately after harvest of the plants because of the possibility of the contamination due to the presence of nodules. After 4 months storage it was expected that the rhizobial populations would have stabilized and the influence of pH would be evident.

RESULTS AND DISCUSSION

The yield data of arrowleaf clover plants inoculated with different strains of *R. leguminosarum* biovar *trifolii* (Fig. 1) are ranked by increasing yield response of plants grown at pH 6.8. Growth of arrowleaf clover was influenced by a significant interaction ($P = 0.05$) between the strain of *Rhizobium* used as inoculum and soil pH. Variability in symbiotic effectiveness was expressed among the strains at each pH. Plants grown at pH 6.8 and inoculated with isolate 6A yielded significantly more than plants inoculated with all other strains, with the exception of strain 162X68. Inoculated plant yields ranged from 70 to 45% of the plus-N uninoculated plants at pH 6.8, indicating that the symbiotic system was not providing adequate N.

Yield of inoculated plants grown at pH 5.6 was considerably lower than plants grown at pH 6.8 (Fig. 1). Uninoculated plants supplied with mineral N again outyielded all inoculated plants (Fig. 1). Dry wt of inoculated plants was highest but not significantly different for strains 162X68, 162X97, 6A and 162K13. Plants inoculated with these strains yielded approx. 45% of the plus-N control plants. At pH 5.6,

Table 1. Chemical characteristics of the Bowie Variant (Glossic Paludalf fine-loamy, siliceous, thermic) after adjustment to three pH regimes

pH	P	Ca	Mg	K	Na	Zn	Fe	Cu	Mn
	(mg kg^{-1} soil)								
6.8	33	548	58	260	175	2.0	10	0.36	3.2
5.6	26	217	61	316	210	2.0	13	0.30	5.7
5.0	26	217	55	288	140	2.0	13	1.28	6.4

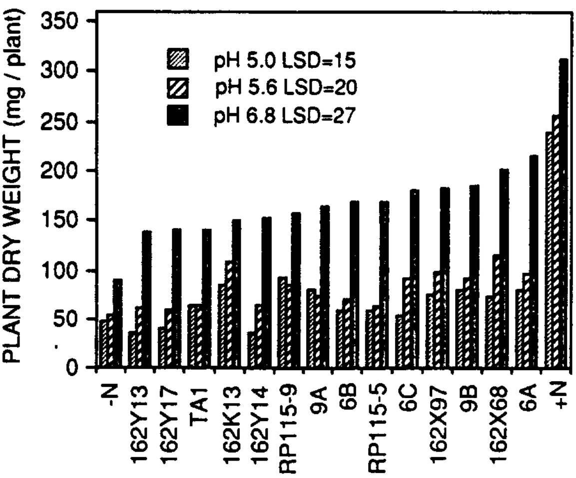

Fig. 1. Dry wt of arrowleaf clover grown in pots of soil at pH 5, 5.6 and 6.8. Plants were inoculated with different strains of rhizobia and grown in a glass house for 52 days.

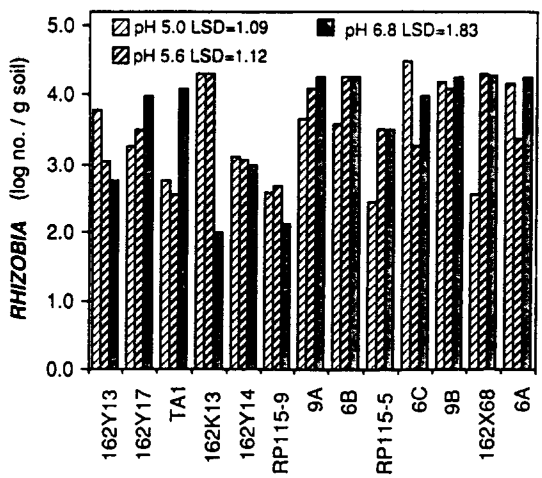

Fig. 2. Populations of rhizobia in pH 5, 5.6 and 6.8 soil, 5 months following the harvest of arrowleaf clover.

plants inoculated with seven of the strains yielded no more than the uninoculated no-N control, whereas at pH 6.8 all inoculated plants yielded more than the no-N control. Roots were visually inspected for nodules at harvest and all inoculated plants were well nodulated, whereas the uninoculated plants were not nodulated.

Yield of plants grown on the pH 5 soil was similar to or lower than plants grown on the pH 5.6 soil with an overall mean 65 vs 81 mg plant^{-1}, respectively. Again, the uninoculated plants supplied with N had much larger yields than by inoculated plants. The best yield of the inoculated treatments at pH 5 was 92 mg for plants inoculated with strain RP115-9, which was equal to only 38% of the plus-N uninoculated plants. A comparison of plants grown at pH 6.8 and 5 demonstrates that the dry wt of N-fertilized plants only declined by 22% while the average dry wt of inoculated plants decreased by 62%. Andrew (1976) observed a 60% reduction in dry matter yield of inoculated *T. repens* when grown at pH 4.5 vs 6 in sand culture, but the yield of plants supplied with inorganic N was only reduced by 20%.

Plants grown at pH 5 and 5.6 contained high concentrations of Mn (Table 2). Data for inoculated plants were averaged at each pH because the Mn contents were similar. The concentration of Mn in shoots of both inoculated and uninoculated plants increased as the soil pH decreased. Even though uninoculated plants that received mineral N had high tissue concentrations of Mn at pH 5 and 5.6, these plants grew well.

Table 2. Concentration of Mn in shoots of uninoculated +N, and inoculated arrowleaf clover plants grown on a Bowie soil at three pH regimes

	Soil pH		
	5.0	5.6	6.8
Treatment	(mg Mn kg^{-1} dry matter)		
Uninoculated (+N)	744	366	156
Inoculated	768	524	194
SD	12	28	44

The standard deviation was calculated for three samples of inoculated plants at each pH.

Data are not available on toxic concentrations of Mn in arrowleaf clover. Incipient Mn toxicity was found in other *Trifolium* spp having Mn concentrations of 500–650 mg kg^{-1} (Andrew and Hegarty, 1969). Mn concentration in the tops of *Medicago* spp experiencing toxicity ranged between 500 and 700 mg kg^{-1} while the tolerant subclover (*T. subterraneum* L.) tissue accumulated less than 200 mg kg^{-1} (Robson and Loneragan, 1969). In our experiment, growth of arrowleaf clover dependent on symbiotic dinitrogen fixation in an acid soil was reduced at a tissue concentration of 540 mg Mn kg^{-1} but yield reduction was confounded with acidity. A higher concentration of Mn would be required to greatly reduce growth of plants supplied with mineral N (Fig. 1 and Table 2).

Populations of *R. leguminosarum* biovar *trifolii* in the soils 5 months after harvest were influenced by the interaction of strain of rhizobia and pH (Fig. 2). Increases in soil acidity were not consistently associated with decreases in rhizobial numbers. Some strains had higher populations in the more acid soils. In general, survival of the isolates from acid soils (6A, 6B, 6C, 9A and 9B) was better than for the commercial strains and was not influenzed by reducing the soil pH. Nevertheless, the size of the populations for any strain would be considered adequate for nodulation.

Our investigation demonstrates that strains of rhizobia differ in their ability to sustain plant growth on an acid soil with potentially toxic concentrations of Mn, and survival of the rhizobia did not appear to be a problem. Arrowleaf clover was capable of growing at pH 5 and 5.6 when provided with mineral N. Nodule function appears to be limited by acidic conditions and/or relatively high concentrations of Mn. Investigations need to be undertaken to determine the mechanisms of Mn or acidity to symbiotic dinitrogen fixation. The large yield depression that occurred for all strains indicate that rhizobial strain selection alone may not provide a solution to the problem. Perhaps plant selection in conjunction with strain selection would provide a viable solution.

REFERENCES

Andrew C. S. (1976) Effect of calcium, pH and nitrogen on the growth and chemical composition of some tropical and temperate pasture legumes. I. Nodulation and growth. *Australian Journal of Agricultural Research* **27**, 611–623.

Andrew C. S. and Hegarty M. P. (1969) Comparative responses to Mn excess of 8 tropical and 4 temperate pasture legume species. *Australian Journal of Agricultural Research* **20**, 687–696.

Dobereiner J. (1966) Manganese toxicity effects on nodulation and nitrogen fixation in beans (*Phaseolus vulgaris* L.) in acid soils. *Plant and Soil* **24**, 153–166.

Evers G. W. (1985) Clover establishment and growth at different pH levels. *Forage Research in Texas, 1985*, pp. 46–48. Consolidated Progress Report 4347. The Texas Agricultural Experiment Station, College Station.

Foy C. D. (1984) Physiological effects of hydrogen, aluminum, and manganese toxicities in acid soil. In *Soil Acidity and Liming* (F. Adams, Ed). *Agronomy Monograph 12.* American Society of Agronomy, Madison.

Holding A. J. and Lowe J. F. (1971) Some effects of acidity and heavy metals on the *Rhizobium*-leguminous plant association. *Plant and Soil* Special Volume, 153–166.

Hoveland C. S., Carden E. L., Buchanan A., Evans E. M., Antony W. B., Mayton E. L. and Burgess H. E. (1969) Yuchi arrowleaf clover. *Alabama Agricultural Experiment Station Bulletin 396*, Auburn.

Keyser H. H. and Munns D. N. (1979) Tolerance of rhizobia to acidity aluminum, and phosphate. *Soil Science Society of America Journal* **43**, 519–523.

Keyser H. H., Munns D. N. and Hohenberg J. S. (1979) Acid tolerance of rhizobia in culture and in symbiosis with cowpea. *Soil Science Society of America Journal* **43**, 719–722.

Lowe J. F. and Holding A. J. (1970) Influence of clover source and of nutrient manganese concentration on the *Rhizobium* white clover association. In *White Clover Research, Occasional Symposium No. 6 British Grassland Society* (J. Lowe, Ed), pp. 61–70. Inprint, Luton.

Munns D. N. (1978) Soil acidity and nodulation. In *Mineral Nutrition of Legumes in Tropical and Subtropical Soils* (C. S. Andrew and E. J. Kamprath, Eds), pp. 247–263. CSIRO, Melbourne.

Olsen S. R. and Sommers L. E. (1982) Phosphorus. In *Methods of Soil Analysis, Part 2. Chemical and Microbiological Properties* (A. L. Page, R. H. Miller and D. R. Keeney, Eds), pp. 403–430. American Society of Agronomy, Madison.

Parkinson J. A. and Allen S. E. (1975) A wet oxidation procedure suitable for the determination of nitrogen and mineral nutrients in biological material. *Communications in Soil Science and Plant Analysis* **6**, 1–11.

Robson A. D. and Loneragan J. F. (1969) Sensitivity of annual *Medicago* species to manganese toxicity as affected by calcium and pH. *Australian Journal of Agricultural Research* **21**, 223–232.

Vose P. B. and Jones D. G. (1963) Interaction of manganese and calcium on nodulation and growth of *Trifolium repens*. *Plant and Soil* **18**, 372–385.

Weaver R. W. and Frederick L. R. (1982) *Rhizobium*. In *Methods of Soil Analysis, Part 2. Chemical and Microbiological Properties* (A. L. Page, R. H. Miller and D. R. Keeney, Eds), pp. 1043–1070. American Society of Agronomy, Madison.